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Accuracy of serological testing for SARS-CoV-2 antibodies: first results of a large mixed-method evaluation study

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Abstract

Background: Serological immunoassays that can identify protective immunity against SARS-CoV-2 are needed to adapt quarantine measures, assess vaccination responses, and evaluate donor plasma. To date, however, the utility of such immunoassays remains unclear. In a mixed-design evaluation study, we compared the diagnostic accuracy of serological immunoassays that are based on various SARS-CoV-2 proteins and assessed the neutralizing activity of antibodies in patient sera.

Methods: Consecutive patients admitted with confirmed SARS-CoV-2 infection were prospectively followed alongside medical staff and biobank samples from winter 2018/2019. An in-house enzyme-linked immunosorbent assay utilizing recombinant receptor-binding domain (RBD) of the SARS-CoV-2 spike protein was developed and compared to three commercially available enzyme-linked immunosorbent assays (ELISAs) targeting the nucleoprotein (N), the S1 domain of the spike protein (S1) and a lateral flow immunoassay (LFI) based on full-length spike protein. Neutralization assays with live SARS-CoV-2 were performed.

Results: One-thousand four-hundred and seventy-seven individuals were included comprising 112 SARS-CoV-2 positives (defined as a positive real-time PCR result; prevalence 7.6%). IgG seroconversion occurred between day 0 and day 21. While the ELISAs showed sensitivities of 88.4% for RBD, 89.3% for S1, and 72.9% for N protein, the specificity was above 94% for all tests. Out of 54 SARS-CoV-2 positive individuals, 96.3% showed full neutralization of live SARS-CoV-2 at serum dilutions ≥1:16, while none of the 6 SARS-CoV-2 negative sera revealed neutralizing activity.

Conclusions: ELISAs targeting RBD and S1 protein of SARS-CoV-2 are promising immunoassays which shall be further evaluated in studies verifying diagnostic accuracy and protective immunity against SARS-CoV-2.

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Introduction

Governments worldwide are facing a unique challenge: to save thousands of lives threatened by coronavirus disease 2019 (COVID-19), while minimising economic and social damage caused by lockdown and other strict measures. Serological immunoassays will play a central role in addressing these challenges for the following reasons[1]. First, serological tests might improve the rate of diagnosis as real-time RT-PCR is associated with a high number of false-negative results due to pre-analytical and other issues[2]. Second, antibody assays may support intensive surveillance measures such as universal testing, active case-finding, contact tracing, and linking clusters and thereby may facilitate an exit strategy from lockdown[3-6]. Third, immunoassays are important to identify individuals with previous severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection who have recovered and to enable adjustment of quarantine measures and physical distancing. Fourth, medical staff with protective immunity against SARS-CoV-2 might be identified and allocated to critical tasks with highly contagious patients. And fifth, vaccination responses in clinical studies could be more effectively monitored. Thus, global authorities including the World Health Organisation strongly encourage studies that investigate performance and application of serological tests for COVID-19.

Various receptors including the angiotensin-converting enzyme 2 (ACE2) expressed by epithelial barrier tissues and immune cells play an important role in SARS-CoV-2 infection as they represent a crucial entry point for the virus[7-9]. The sustained inflammatory immune response caused by SARS-CoV-2 infection contributes the development of clinical manifestations of COVID-19 and the related prothrombotic state[9,10]. In patients with severe disease extensive activation of cytokine-secreting cells from the innate and adaptive immune system has been reported to result in a cytokine storm contributing to acute respiratory distress syndrome and multiorgan failure[11-15]. Antibody responses against different SARS-CoV-2 antigens have been described in serological samples of infected patients. Few patients with anti-viral antibodies have been identified in the first 5 days following symptom onset but the positive rate rapidly increases thereafter[16,17]. To date, antibody testing has focused primarily on two highly abundant structural antigens of SARS-CoV-2, specifically the nucleoprotein (N) protein

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and the spike (S) protein[18]. While the N phosphoprotein ensures the linkage of the viral RNA to the membrane[19], the S glycoprotein binds to ACE2 and thereby initiates viral entry into the host cell[13,20-22]. Neutralizing antibodies (NAb) are typically generated against the S protein and often target the receptor binding domain (RBD)[23,24]. As demonstrated in a vaccination approach using inactivated virus, the RBD represents an immunodominant viral antigen since at least half of the detectable anti-S IgG antibodies were directed against the RBD[25]. In contrast, the amount of anti-N antibodies was 30-fold lower.

Lateral flow immunoassays (LFI)[26,27] as well as enzyme linked immunosorbent assays (ELISA)[28,29] have been developed but not yet adequately evaluated. While LFIs are remarkably fast and only require minutes to perform, significant concern regarding their sensitivity and specificity has been raised[30]. ELISAs are considered more robust but require highly specialized laboratories with the capacity to run automated high-throughput measurements.

At the time of compiling this paper, the diagnostic performance of different immunoassays as well as their predictive value for protective immunity remains unclear. Before a broad implementation of immunoassays can be justified, the following points need to be carefully assessed in adequately powered and designed diagnostic studies: (1) diagnostic accuracy (or sensitivity/ specificity respectively) in the acute and subacute phase of the disease, (2) antibody kinetics over time in patients with confirmed COVID-19, (3) extent of cross-reactivity with other pathogens and patients with autoimmune disorders, (4) reliability between different assay settings and material characteristics, as well as (5) correlate of protective immunity[3].

With the present study, we aimed to comprehensively establish the utility and diagnostic accuracy of serological immunoassays for SARS-CoV-2 infection and to explore protective immunity[12] as predicted by such immunoassays in a mixed-method observational study of hospital inpatients as well as medical personnel.

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Methods

Study design, setting and population

International guidelines on study design were strictly followed[31] and cross-sectional, prospective observational, as well as case-control designs were used. Participants were recruited via three different routes: (i) inpatients with a SARS-CoV-2 test result (real-time PCR; RT-PCR), (ii) medical personnel of the Inselspital, and (iii) residual material from patients stored at the Liquid Biobank Bern (www.biobankbern.ch). Inclusion criteria of inpatients are (i) hospitalisation in Inselspital, (ii) tested positive for SARS-CoV-2 using RT-PCR (nasopharyngeal swab), (iii) aged 18 or older and (iv) signed general consent (exemption was granted for a few patients). For this manuscript, only inpatients who had tested positive for SARS-CoV-2 with more than 4 days of residual material available were considered. The temporal pattern of antibody response and seroconversion rate was assessed in a subgroup of inpatients; the first 25 consecutive patients were selected. Inclusion criteria of medical personnel were (i) medical staff at Inselspital since February 2020, (ii) aged 18 or older, and (iii) signed informed consent. The personnel were recruited via mailing lists. A limited number of fully anonymized, residual biobank samples were also used for the purpose of this study with the inclusion criterion of having been collected from inpatients between December 2018 and February 2019. A total of 54 randomly selected sera from individuals who were tested positive in either of the three ELISA immunoassays as well as 6 negative controls were assessed in a live SARS-CoV-2 neutralization assay (all collected in April 2020).

The University Hospital Bern (Inselspital) is one of the largest tertiary hospitals in Switzerland covering a catchment area of more than 1 million inhabitants. With several associated smaller hospitals, it provides the full spectrum of general as well as highly specialised medical services. More than 10,000 employees work at the Insel Gruppe AG.

The study was supported by the local COVID-19 task force. The study protocol was approved by the appropriate ethics committee and the authorities of the University Hospital and conducted in accordance with the Declaration of Helsinki. The manuscript was prepared according to the Standards for Reporting Diagnostic accuracy studies (STARD) guideline[32].

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Handling of samples and collection of data

Blood was taken following an established in-house protocol to ensure adequate preanalytical conditions and samples were collected using plastic syringes (serum or lithium heparin respectively, S-Monovette®, Sarstedt, Nümbrecht, Germany). Only residual material was used in the case of inpatients. Two tubes (serum and lithium heparin respectively) were drawn in the case of medical personnel. Samples were immediately transported to the central laboratory, processed using a GLP laboratory track and centrifuged within 30 minutes with an established protocol[33].

With regard to inpatients, pseudonymized demographical, clinical as well as laboratory data were extracted and transferred by the Insel Data Science Center (IDSC) from electronic patient documentation. Limited data were collected for the purpose of this sub-study: age, gender, time interval since RT-PCR (nasopharyngeal swab). A positive SARS-CoV-2 RT-PCR result was used as additional inclusion criterion. With regard to medical personnel, a RedCap database survey was constructed collecting demographical data, COVID-19 symptoms (presence, extent and date), comorbidities and risk factors, professional exposure, and date of RT-PCR.

Generation of recombinant RBD protein

The S1 protein and RBD are regarded as ideal candidates for the development of diagnostic tests and vaccines targeting SARS-CoV-2[34]. The pCAGGS plasmid containing the human codon-optimized sequence of the SARS CoV-2 S protein receptor binding domain (RBD, amino acids R319-F541) with native S signal sequence (amino acids M1-S14) and a C-terminal hexahistidine tag was kindly provided by Prof. Florian Krammer. Plasmid DNA was prepared using the Gene Elute HP Plasmid Maxiprep Kit (Sigma-Aldrich). Prior to transfection Expi293F cells (Thermo-Fisher) were grown to a density of 3.0 x 10^6 cells/ml in culture medium (a mixture of 33% Expi293 and 66% FreeStyle-293 media from Thermo-Fisher). For each liter of transfection, 0.5mg of plasmid DNA was diluted in 100ml of culture medium, mixed with 1.3ml FectoPro transfection reagent (Polyplus), and incubated for 10 minutes at room temperature prior to addition to cells. Immediately following transfection cells were supplemented with 100x D-glucose (400g/l)

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and 100 x Valproic acid (300mM) boost solutions. Three days post transfection the cell culture supernatants were harvested by centrifugation at 7,000 x g for 15min. Supernatants were passed through a 0.22µm filter and 1:1 diluted with PBS containing 10 mM imidazole. For purification of his-tagged RBD protein 5 ml Ni-NTA resin (HisPur NiNTA ThermoFisher) was washed three times with washing buffer (PBS with 10mM imidazole) and incubated on a stir plate at 4°C for 1 hour. Subsequently, the mixture was poured into a glass column with a frit and washed 3 times with 5 column volumes of washing buffer. The protein was then eluted three times with 15 ml PBS containing 250mM imidazole. Elutions were pooled and dialyzed overnight against PBS using 3.5 kDa cutoff SnakeSkin dialysis tubing. The final protein concentration was determined by NanoDrop measurement at A_{280}. The quality of recombinant RBD protein was analyzed by SDS-PAGE and analytical size-exclusion chromatography.

Development of an in-house ELISA

All ELISA assays were performed on a DSX automated ELISA system device (DYNEX Technologies). The in-house assay was prepared as follows: 96-well plates were coated overnight at 4°C with 100µl of 1µg/ml RBD protein in PBS. The following day, each well was blocked with 300µl of PBS/0.15% casein at 4°C until use and at least overnight. Subsequently plates were washed twice with PBS and 100µl sera were added at a 1:100 dilution in PBS/0.15% casein for 1 hour at RT. After five washes with 300µl PBS/0.1% Tween 100µl of HRP-labeled secondary polyclonal anti-human IgM (Sigma, A0420) and anti-human IgG (Sigma, A0170) antibodies were added in a 1:10'000 dilution for 30 minutes at RT. Again, the plates were washed 5 times with PBS/0.1% Tween and 100µl of TMB substrate solution (Sigma, T4444) was added for 15 minutes at RT. The development was stopped by adding 100µl of 0.5M H_2SO_4 and results were measured at OD450-620nm. All samples with an OD > 0.5 were assigned as positive.

Determination of commercially available immunoassays

Several commercial tests were conducted according to the manufacturers' instructions. An ELISA produced by Euroimmun AG, Lübeck, Germany targeting the S1 protein as the

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immobilized antigen for the detection of IgG antibodies was employed. Briefly, samples were diluted 1:100 in sample buffer and 100µl of diluted samples, pre-diluted positive and negative controls, as well as pre-diluted calibrator were added for 1 hour at 37°C. After three wash steps with 300µl wash buffer, 100µl of HRP-labeled secondary anti-human IgG antibodies were added for 30 minutes at 37°C. The plates were washed again three times with wash buffer and 100µl of TMB solution was added for 20 minutes at RT. The development was stopped by adding 100µl of 0.5M H_2SO_4 and results were measured at OD450-620nm. Antibody values were expressed as a ratio ($OD_{sample}/OD_{calibrator}$). All samples with a ratio >1.1 were assigned as positive.

A different ELISA determining IgG and IgM antibodies against N (Epitope Diagnostics Inc., San Diego, CA) was used as follows. For IgG, samples were added to the immobilized antigen in a 1:100 dilution (in IgG sample diluent) for 30 minutes at RT. After washing the plates five times with diluted wash concentrate, 100µl of HRP-labeled secondary anti-human IgG antibodies were added for 30 minutes at RT. The plates were washed again five times with diluted wash concentrate and 100µl of TMB solution was added for 20 minutes at RT. The development was stopped by adding 100µl of 0.5M H_2SO_4 and results were measured at OD450-620nm. Cut-off for a positive reaction was calculated by the following formula: 1.1*(mean $OD_{Negative\ Control}$ + 0.18). For IgM, 10µl of undiluted samples were added to the immobilized anti-IgM in the microtiter plate, and 100µl of IgM Sample Dilution buffer were added and incubated at 37°C for 30 minutes. After washing the plates five times with diluted wash concentrate, 100µl of HRP-conjugated SARS-CoV-2 antigen was added for 30 minutes at 37°C. The plates were washed again five times with diluted wash concentrate and 100µl of TMB solution was added for 20 minutes at RT. The development was stopped by adding 100µl of 0.5M H_2SO_4 and results were measured at OD450-620nm. Cut-off for positive reaction was calculated by the following formula: 1.1*(mean $OD_{Negative\ Control}$ + 0.10).

A lateral flow rapid test determining IgG and IgM antibodies against the recombinant S protein (Autobio Diagnostics Co, LTD, Zhengzhou, China) was used according the instructions. Briefly, 5µl of serum or heparin plasma, or alternatively, 10µl of whole capillary blood were added to both IgM and IgG cavities of the cassette. 60µl of reaction

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buffer were added and reactions were visually read after 15 minutes and assessed as negative, equivocal or positive reactions.

Determination of real-time PCR

Patient sample material was obtained by performing nasopharyngeal swabs using Copan FLOQSwabs and Copan UTM Viral Transport medium (Copan, Brescia, Italy). Collected patient sample material was transported to the laboratory at room temperature and stored at 4°C until processing. For nucleic acid testing (NAT), three different methodologies were used in the respective time period of sample collection. An laboratory developed test (LDT) RT-PCR workflow based on the published protocol of Corman *et al.* was utilised for pan-Sarbecovirus E-gene detection[35] followed by the detection of the SARS-CoV-2 specific RdRP-gene. Additionally, two commercial, fully automated workflows, the Seegene Allplex 2019-nCoV Assay (Seegene, Seoul, Korea) and the Roche cobas® SARS-CoV-2 Assay (Roche Diagnostics, Rotkreuz, Switzerland) were used.

RNA for the LDT RT-PCR workflow was extracted after inactivation of patient samples using AVL buffer (Qiagen, Hilden, Germany) in a ratio of 200 µl sample + 800 µl buffer using the MagNA Pure 24 system (Roche Diagnostics, Rotkreuz, Switzerland) or ELITe InGenius SP 1000 extraction cassette on an InGenius System (Elitech, Torino, Italy) and eluted in 100 µL. Alternatively, an adapted cobas 4800 workflow used the extraction chemicals and protocol for the IVD workflow for the cobas HIV-1 Assay for the cobas® 4800 System (Roche Diagnostics, Rotkreuz, Switzerland) in which 400 µL non-inactivated patient sample eluted in 100 µL was performed. RNA for the Allplex 2019-nCoV workflow was extracted according to the manufacturer's instructions using Seegene's STARlet IVD workflow. RNA for cobas® SARS-CoV-2 was extracted according to the manufacturer's instructions using Roche's cobas® 8800 system.

Seegene Allplex 2019-nCoV and cobas® SARS-CoV-2 were performed according to the manufacturer's instructions using a STARlet IVD System or a cobas 8800 system, respectively.

For the LDT E-gene and RdRP-gene detection the following was used: RT-PCR assays RNA Process Control Kit (Roche Diagnostics, Rotkreuz, Switzerland; Cat. No. 07 099

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592 001) containing LightCycler Multiplex RNA Virus Master for reverse transcription / amplification, RNA Process Control as an internal control for extraction and PCR and RNA Process Control Detection Assay (proprietary Cy5-labeled probe and primers) for RNA Process Control detection. A 20 µL reaction contained 5 µL of RNA, 1.0 µL of 20x RNA Process Control Detection Assay, 0.1 µL of 200x RT Enzyme Solution and 4.0 µL of 5x RT-qPCR Reaction Mix. For E-gene detection, primer/probe sequences and concentrations were used according to the published protocol[35]. Primer/probe sequences and concentrations used for the LDT-RdRP Assay are as follows: RdRP1-F1; AAATGGTCATGTGTGGCGGT; 900 nM / RdRP1-R2; ATTAACATTGGCCGTGACAGCT; 900 nM / RdRP1-P3mgb; FAM-CTCATCAGGAGATGCC-MQ530; 100 nM / RdRP2-F4; ATGGTCATGTGTGGCGGC; 900 nM / RdRP2-R5; GCATTTACATTGGCTGTAACAGCT; 900 nM / RdRP2-P6mgb; HEX-CATCATCCGGTGATGCT-MQ530; 100 nM. Primer/probe system RdRP1 is specific for detection of SARS-CoV-2 and primer/probe system RdRP2 is specific for detection of SARS-CoV-1. All oligonucleotides were synthesized and provided by Mycrosynth AG (Balgach, Switzerland). Thermal cycling was performed at 50 °C for 10 min for reverse transcription, followed by 95 °C for 30 sec and 45 cycles of 95 °C for 5 s, 60 °C for 30 s. A Roche Light Cycler 480 instrument was used for amplification and fluorescence detection (Roche Diagnostics, Rotkreuz, Switzerland).

Definition of diagnoses

As the primary reference standard test, a positive RT-PCR test from a nasopharyngeal swab was defined as 'confirmed SARS-CoV-2 infection' (SARS-CoV-2+). In light of the limitations of this test, it was used as the only reference standard for the purpose of this first analysis. Sensitivity analyses using additional reference standards will be employed in subsequent phases of this study (focusing on different clinical manifestations). 'SARS-CoV-2 negative' (SARS-CoV-2-) was defined as (i) a negative RT-PCR result in all nasopharyngeal swabs conducted, *or* (ii) RT-PCR not performed (because all patients and medical staff would have been tested in case of symptoms according to applicable regulations). Biobank samples from Winter 2018/2019 were also categorized as negative. Comorbidities and risk factors, which will be used as covariables in subsequent phases of this study, will be extracted from electronic patient records and asked in the RedCap

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survey of medical staff, assuming that classifications have been made according to current guidelines.

Live SARS-CoV-2 neutralization assay

Serum samples were tested for the presence of neutralizing antibodies determined by the inhibition of virus-induced cytopathic effect (CPE). Briefly, sera from 56 SARS-CoV-2 positive individuals (inpatients as well as medical personnel) and 6 sera from SARS-CoV-2 negative individuals (medical personnel) were incubated at 56°C for 30 min for complement inactivation, centrifuged at 13,000 rpm for 10 min and diluted 1:8 in cell culture medium (MEM Eagle with EBSS, 20mM HEPES with 0.85 g/l NaHCO3, supplemented with 2% FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine (2 mmol/l); all from Bioswisstec AG, Schaffhausen, Switzerland). Of the 1:8 serum dilutions, 112.5 µl were transferred into a 96-well plate in duplicates. From there, 12.5 µl was used to produce a 5-fold serial dilution in 50 µl culture medium; 50 µl was transferred to another row to control for serum toxicity on control cells. Then, serially diluted sera were mixed with 50 µl/well of diluted virus culture corresponding to 100 TCID50 of infectious SARS-CoV-2 (BetaCoV/France/IDF0372/2020). The serum/virus mixture was incubated for 1 h at 37°C and subsequently transferred to a confluent 96-well-plate with Vero-E6 cells. Plates were incubated for 3 days at 37°C with CO2 bags and CPE was determined by crystal violet staining. Wells which showed no CPE as judged by a complete cell layer were rated (-) and wells showing signs of CPE were rated (+). Full neutralization titer was determined as the serum dilution were no signs of CPE (-) was observed in both duplicates. The strain BetaCoV/France/IDF0372/2020 was supplied by the National Reference Centre for Respiratory Viruses hosted by Institut Pasteur (Paris, France) and headed by Pr. Sylvie van der Werf. The human sample from which strain BetaCoV/France/IDF0372/2020 was isolated has been provided by Dr. X. Lescure and Pr. Y. Yazdanpanah from the Bichat Hospital, Paris, France. Moreover, the strain BetaCoV/France/IDF0372/2020 was supplied through the European Virus Archive goes Global (Evag) platform, a project that has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 653316.

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Statistical analysis

Descriptive statistics have been used to illustrate the distribution of immunoassay test results in patients and medical staff with and without SARS-CoV-2 infection and to show temporal patterns. Days until seroconversion were plotted following the occurrence of symptoms or a positive RT-PCR result, respectively. To calculate measures of diagnostic accuracy, we pooled all patients and medical personnel being aware that the composition of this cohort is artificial. The time point since RT-PCR or symptoms respectively was not taken into account because criteria for a clear cut off are lacking. Two-by-two tables were generated, sensitivities and specificities calculated, and receiver-operating-characteristics (ROC) curves plotted as well as c-statistics performed for comparative purposes. A formal power analysis has not been performed, but we have included as many inpatients and medical staff as possible for the purpose of this preliminary analysis and generated confidence intervals as appropriate. Analyses were carried out using the Stata 14.2 statistical software (StataCorp. 2014. Stata statistical software: Release 14. College station, Tx: StataCorp LP). Figures were created using Prism 6 (GraphPad Software, Inc., LaJolla, California, United States).

Results

Characteristics of participants

At University Hospital Bern we first established a carefully designed mixed-method diagnostic accuracy study (**Fig. 1**). Forty-two inpatients with confirmed SARS-CoV-2 infection as defined by a positive RT-PCR (nasopharyngeal swab) with more than 4 days of residual material were available and 202 data points have been analyzed. Median age was 66 years (range 24 to 86), 21% of the patients were female. The median number of days of symptoms before hospitalisation was 7 (range 0 to 18); 48% of the patients were admitted to the intensive care unit. More detailed patient characteristics are reported in **supplementary Table 1**. We additionally included the first 1403 individuals who participated in the cohort of medical staff for the purpose of this analysis. Out of these individuals, 70 were positive for SARS-CoV-2 in RT-PCR, and 75% of SARS-CoV-2 positive individuals (n=53) reported COVID-19 associated symptoms (fever,

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breathlessness, coughing, or loss of smell). The median age was 39 (range 23, 65), 60.7% of the individuals were women (n=851). Further, we included residual serum samples of patients stored at the Liquid Biobank Bern (n=32). These samples were frozen between December 2018 and February 2019 before the global outbreak of SARS-CoV-2 infections.

Analytical characteristics of in-house ELISA (RBD)

Recombinantly expressed RBD has been used to establish an in-house ELISA for the detection of IgM and IgG anti-SARS-CoV-2 antibodies in human serum samples (**supplementary Fig. 1a,b**). Optimal serum dilutions were determined by titration of sera derived from six SARS-CoV-2+ and six SARS-CoV-2- individuals. The serum dilution of 1:100 allowed efficient discrimination between positive and negative outcome (**supplementary Fig. 2**). After automatization on a DYNEX DSX device, the intra-assay (within-run) and inter-assay (day-to-day) precisions of the in-house RBD ELISA was assessed (**supplementary Fig. 3a-d**, **supplementary Table 2a,b** and **supplementary Table 3a,b**). We further compared serum and heparin-plasma (**supplementary Fig. 3e,f)**. Overall, the in-house RBD ELISA assay showed high intra- and inter-assay reproducibility and demonstrated a high degree of agreement between plasma and serum samples.

Temporal pattern of antibody response as measured by different immunoassays

Among a subgroup of 25 SARS-CoV-2+ inpatients, seroconversion for IgM and IgG antibodies was observed between day 0 and day 21 after the RT-PCR result and between day 2 and day 21 after the start of symptoms (**Fig. 2**). Interestingly, IgM and IgG antibody responses against RBD and S1 were substantially more pronounced as compared to N. Assessment of the longitudinal dynamics of patient sera revealed a marked and consistent increase of IgG antibodies for RBD and S1 (**Fig. 3a**). IgM antibodies were measured in the RBD and N ELISA and detectable at least for two weeks after seroconversion (**Fig. 3b**). Interestingly, the individual temporal IgG and IgM patterns showed a high degree of inter-individual variability with one group of patients

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displaying high antibody responses already at the time of hospitalization, while a second group seroconverted within the first week of hospitalization and a third group that mounted only a week response within the time of analysis (**supplementary Fig. 4** and **supplementary Fig. 5**). Additionally, we analysed serum samples from 32 inpatients between December 2018 and February 2019 (biobank samples from winter 2018/2019). Of these samples, all were negative for anti-RBD IgM and IgG, as well as anti-S1 IgG. However, two biobank samples tested positive for anti-N IgG (ELISA; 6.2%), and one tested positive for anti-N IgM (ELISA; 3%). All samples were negative for anti-S IgG and IgM (100%) as tested by LFI.

Diagnostic accuracy

The pooled study population consisted of 1477 individuals, 112 of whom tested as RT-PCR positive (prevalence 7.6%). Sera from all individuals were tested in the three different ELISA setups for IgG and IgM anti-SARS-CoV-2 antibodies (**Fig. 4a**). A subgroup of samples (n=159) was additionally assessed on LFI (**Fig. 4b**). Both assay formats showed high specificity above 94% for IgG and IgM measurements (**supplementary Table 4**). However, the sensitivity between assays and formats varied considerably. The highest sensitivities were reached for IgG measurements with the S1 (89.3%) and RBD (88.4%) ELISA, followed by IgG measurements on N (72.9%) ELISA. Sensitivities for IgM measurements were all considerably lower for both ELISA and LFI formats, which could be due to the more transient detectability of IgM upon infection.

To detect potential sources of variability, we additionally studied the antibody response in salient subgroups of RT-PCR positive individuals (**Fig. 4c)**. First, higher IgG antibodies were found in inpatients compared to medical personnel, potentially reflecting the higher proportion of patients with severe disease (RBD ELISA; mean OD difference 0.44; SD 0.19; p=0.01). Second, no significant difference was found between individuals with and without COVID-19 symptoms (mean OD difference 0.11; SD 0.24; p=0.64). Third, the IgG response was higher in hospitalized patients compared to outpatients (0.50; SD 0.18; p=0.005), but not in ventilated patients compared to hospitalized patients (mean OD difference 0.08; SD 0.23; p=0.72). Fourth, IgG response was higher in patients above 50 years than patients below (0.54; SD 0.25; p=0.03).

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In the tested inpatient population, we observed three “false-negative” (negative in S1 ELISA despite positive RT-PCR) outcomes. Among three false-negative inpatients (P07, P041, and P042), two were measured at an early time-point (Patient 7 and 41), and one patient (P042) might have experienced seroconversion at a very late time-point because of a significant increase of antibody titers at day 24 (**supplementary Fig. 3** and **supplementary Fig. 4**). In the assessed hospital staff, seven were classified as “false-negative”. All of these reported mild diseases and had symptoms clearly associated with COVID-19 (fever, breathlessness, cough, and loss of taste or smell). Twenty-two individuals in the hospital staff group tested “false-positive” (positive S1 ELISA results despite negative RT-PCR). Fourteen of them experienced one or more symptoms clearly associated with COVID-19. The remaining eight individuals were clearly positive in at least three assays. All other individuals were either classified as “true-positive” (positive in S1 ELISA, and positive in RT-PCR), or as “true-negative” (negative in S1 ELISA, and negative in RT-PCR).

In terms of performance, the calculated area under the receiver operating characteristic (ROC) curve for IgG measurements was 0.96 (95% CI: 0.94 to 0.98) in the RBD, 0.98 (95% CI: 0.97 to 0.99) in the S1, and 0.91 in the N (95% CI: 0.86 to 0.95) ELISA (**Fig. 5a**), while for IgM measurements it reached 0.86 (95% CI: 0.81 to 0.90) for RBD ELISA and 0.84 (95% CI: 0.80 to 0.89) for N ELISA (**Fig. 5b**).

SARS-CoV-2 neutralization corresponds with ELISA positivity

A total of 54 randomly selected sera from individuals who were tested positive in either of the three ELISA immunoassays as well as 6 negative controls were assessed in a live SARS-CoV-2 neutralization assay using ACE2-expressing Vero-E6 cells (34 inpatient samples, and 26 samples of medical personnel). Full neutralization of viral infection has been determined based on 100% inhibition of the cytopathic effect in a serial dilution of the sera (**supplementary Fig. 6**). The means of highest serum dilutions at which full neutralization was observed correlated remarkably well with the measured antibody responses in the ELISA immunoassays (**Fig. 6a-c**). Importantly, 96.3% of the sera from ELISA positive individuals showed full inhibition at serum dilutions ≥1:16. The two sera that did not show neutralization (P037 and P042) were drawn at an early time point

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where the patients did not yet show antiviral antibodies. Both patients, however, fully neutralized the virus after seroconversion at a later time point (**Fig. 6d**). Further, all 6 sera from ELISA negative individuals showed no neutralizing activity. Of note, one or two ELISA assays were negative in 17 samples with full neutralization.

Discussion

We report first results of a large, mixed-design evaluation study which was implemented to compare the diagnostic accuracy of serological immunoassays for SARS-CoV-2 antibodies. While the time to seroconversion varied substantially between infected individuals, the mounted IgG responses were robust and stable over time in all assays relying on RBD, S1 as well as N. With regards to the ELISA assays, the overall diagnostic accuracy was adequate with a high specificity. Some “false-positive” results are likely due to a rather narrow diagnostic window and limited sensitivity of the RT-PCR as well as asymptomatic disease course [36]. “False-negative” results may be caused by a long seroconversion period observed in some patients and mild disease course in other individuals. The accuracy measures of LFI and N were inferior compared to ELISA targeting S1 and RBD. Strikingly, there is a high degree of correlation between antibody responses to these viral surface proteins and the neutralizing activity against live SARS-CoV-2.

A few other studies have previously assessed the diagnostic accuracy of serological immunoassays. Recently, Long and colleagues studied the antibody response in 285 patients with COVID-19 using a magnetic chemiluminescent immunoassay. [37] In accordance with their results, we observed high inter-individual variation in the time to seroconversion. In contrast to their study, we confirmed these findings with an appropriate diagnostic accuracy protocol using different serological immunoassays. In another case-control study, Infantino et al. analyzed 61 COVID-19 inpatient samples and 64 selected patients collected before 2020 using a magnetic chemiluminescent immunoassay[38]. In agreement with their results, we found limited sensitivity but high specificity of the serological SARS-CoV-2 immunoassays. In further study conducted at the Geneva University Hospital, 181 samples of COVID-19 patients were included as well as 176 controls collected before 2020, and analyzed with the same S1 ELISA that we

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used in our study. Similar to our results they report a high specificity for IgG, particularly with an adjusted cut-off value[39]. In line with other studies the accuracy and performance LFIs was rather weak[40,41].

The study presented here adds important value to previous reports as it (i) was designed as a comprehensive diagnostic accuracy study combining different research methods, (ii) directly compares major assay approaches, (iii) was fully approved by all appropriate authorities, (iv) was independently conducted at a University Hospital, (v) includes comprehensive neutralization experiments with live virus and (vi) fully describes all clinical and technical procedures. Additionally, the study design allows straight forward expansion to automated immunoassay testing. On the other hand, a potential shortcoming of this study is the limited number of tested individuals. Besides, the observation period was limited to approximately one month, making it impossible to conclude beyond this time point.

Our findings emphasize that serological immunoassays are an important diagnostic tool in the context of the current COVID-19 pandemic. While positive ELISA test results correctly identified SARS-CoV-2 infected individuals with high probability in our study, the results also revealed that not all cases could be captured. This confirms other studies reporting that many asymptomatic and mild cases of SARS-CoV-2 infections do not lead to a detectable seroconversion [42,43]. In this study, we further confirm that a general timepoint of seroconversion cannot be predicted. However, longitudinal tracing of patients reveals individual seroconversion. So far, the extent of cross-reactivity to other pathogens appears low, but this must as well be addressed in larger patient populations.

Moreover, our results from the live SARS-CoV-2 neutralization assays strongly indicate the presence of protective antibodies in 96.3% of tested sera within the one-month follow-up period assessed in this study. Previous work in non-human primates have reported that serum dilutions of 1:16 were protective for re-infection with SARS-CoV-2[44]. Our findings are in line with previous reports describing a close correlation of antibody responses against RBD, S1 and N protein of SARS-CoV-2 with full neutralizing activity and indicate that such serological tests might even be used to predict protective immunity in near future[45,46]. To draw further conclusions, however, SARS-CoV-2 positive patients have to be followed over an extended time period in future studies.

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In line with previous studies[47], we observed that the antibody response is more pronounced in patients with severe disease than patients without (Figure 4, panel C; inpatients, hospitalized patients, older patients). However, the response was similar in patients with mechanical ventilation and hospitalized patients. This is most likely due to limitations in sensitivity, which does not contradict our general observations.

In summary, we report the first results of a large, mixed-design evaluation study that has been conducted in an independent academic setting at the University Hospital Bern to assess the diagnostic accuracy of various immunoassays to determine antibody responses against SARS-CoV-2. While antibody responses of individual COVID-19 patients against RBD and S1 protein were similar, a weaker reactivity against N protein became apparent. The time to seroconversion varied substantially between COVID-19 patients but the IgG response was robust and stable in all three ELISA setups. Their overall diagnostic accuracy was adequate with a high specificity but limited sensitivity. The antibody responses measured in these ELISAs correlated remarkably well with SARS-CoV-2 neutralizing activity of the sera. On the other hand, accuracy measures of S protein based LFIs were poor. Together, our results emphasize that appropriate serological immunoassays represent a valuable tool to identify a good portion of patients with previous SARS-CoV-2 infection, will help to facilitate exit strategies from lockdown and might even be used to predict immunity to SARS-CoV-2 in near future.

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Authorship details

DB, LFP, AP, PK, TSJ, and AE expressed and analysed recombinant proteins; DB, MPH, AE, and MN developed and validated the RBD immunoassay. PB and HPS developed and implemented the RT-PCR. DS, BW and OE performed virus neutralisation assays. MN developed the study design, the protocol and the analysis plan. TF and CL developed the biobanking solutions. VP, LD, PI, CH, MF and JM contributed to study design and interpretation. PMV, MF, MFB, and SLB provided infrastructure and intellectual reviewed the manuscript. MPH, AE and MN analysed the data and wrote the manuscript.

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Declaration of interests

All authors declare that there is no conflict of interests.

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Figure Legends

Figure 1: Flowchart of study cohort and study design. Only RT-PCR positive inpatients were considered in the current phase of the study (*). Consecutive patients admitted with confirmed SARS-CoV-2 infection were prospectively followed alongside medical staff and biobank samples from winter 2018/2019 (pooled data were used for calculation of diagnostic accuracy). Boxes with a dashed outline are not yet completed. RT-PCR, real-time PCR; ELISA, enzyme linked immunosorbent assay; LFI, lateral flow immunoassay.

Figure 2: Seroconversion rate since symptoms and positive RT-PCR result. The percentage of consecutive patients (n = 25) positively tested for anti-SARS-CoV-2 protein antibodies is shown as a function of time (since symptom onset: red; since positive RT-PCR result: blue line) for IgG in the RBD (a), IgG in the S1 (b), IgG in the N (c), IgM in the RBD (d) and IgM in the N ELISA (e). Curves were calculated using non-linear fitting.

Figure 3: Temporal pattern of antibody responses against SARS-CoV-2 since seroconversion. IgG and IgM antibody responses of consecutive patients (n = 25) as measured by three ELISAs targeting different proteins of SARS-CoV-2: (a) IgG against the receptor binding domain (RBD), the S1 domain of the spike protein (S1) and the nucleoprotein (N); (b) IgM against the receptor binding domain (RBD) and the nucleoprotein (N). Data is shown as mean ± SEM. Curves were calculated using non-linear fitting.

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Figure 4: Distribution of immunoassay results among SARS-CoV-2 positive and negative individuals. Consecutive patients admitted with confirmed SARS-CoV-2 infection were prospectively followed alongside medical staff and biobank samples from winter 2018/2019 (pooled data). (a) IgG and IgM responses against the receptor binding domain (RBD), the S1 domain of the spike protein (S1) and the nucleoprotein (N) of SARS-CoV-2 in SARS-CoV-2 positive (n = 112) and negative (n = 1365) individuals as measured by ELISA. Data are shown as individual data points with a box and whiskers plot indicating minimum to maximum response. (b) IgG and IgM responses against the S1 domain of the spike protein of SARS-CoV-2 as measured by LFI in RT-PCR positive and negative patients. Positive (red), weak positive (green) and negative (blue) responses are shown as percentage of the whole in a pie chart. (c) Antibody response in salient subgroups of RT-PCR positive individuals (inpatients vs. medical personnel, patients with symptoms vs. patients without, hospitalized patients vs. outpatients, patients with ventilation vs. patients without, patients above 50 years vs. patients below 50 years of age).

Figure 5: Accuracy of three different SARS-CoV-2 ELISAs. Receiver operating characteristics curves of (a) IgG and IgM (b) measurements against the receptor binding domain (RBD), the S1 domain of the spike protein (S1) and (c) the nucleoprotein (N) of SARS-CoV-2 in SARS-CoV-2 positive (n = 112) and negative (n = 1365) individuals (N was conducted in 159 individuals only).

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Figure 6: Live SARS-CoV-2 neutralisation. Individual **a**ntibody responses (grey dots) against RBD (a), S1 (b) and N protein (c) in sera of 54 SARS-CoV-2 positive and 6 SARS-CoV-2 negative individuals as measured by ELISA are shown together with the corresponding serum dilution at which full neutralisation of SARS-CoV-2 is observed. Non-linear curve fitting was calculated based on the means of each serum dilution group (red circles). (d) Changes in the serum dilution for full neutralisation of SARS-CoV-2 over time is depicted for seven individual SARS-CoV-2 patients. no NT: no neutralisation detectable.

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Supplementary Figure Legends

Figure S1: Purity and quality check of recombinant RBD protein. (a) SDS-PAGE image of recombinantly expressed and his-tag purified RBD protein. The molecular weight marker (lane 1) is shown next to purified RBD protein (lane 2). (b) An analytical size-exclusion chromatography histogram of purified RBD protein is depicted.

Figure S2: RBD ELISA establishment. Detection of anti-SARS-CoV-2 IgG (a) and IgM (b) antibodies in serially diluted sera of RT-PCR positive (closed circles) and negative (open circles) subjects. Direct comparison of IgG (c) and IgM (d) antibody responses in RT-PCR positive and negative samples at a 1:100 serum dilution. Statistical significance was calculated by Student's *t* test. Data are shown as individual data points with mean values ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure S3: Validation of RBD ELISA. Intra-assay precision analysis of anti-RBD IgG (a) and IgM (b) antibodies with three samples containing different antibody titers at a 1:100 dilution in 10 replicates. The inter-assay precision analysis for anti-SARS-CoV-2 IgG (b) and IgM (d) is shown for a 1:100 diluted serum sample that was run on eight different days in three ELISAs. Anti-RBD IgG (e) and IgM (f) antibodies were measured in serum and plasma from individual patients ($n = 22$). A linear regression analysis was performed.

Figure S4: Anti-SARS-CoV-2 IgG responses of individual COVID-19 patients. IgG responses of individual COVID-19 patients ($n = 25$) against the RBD protein (red), the S1 domain of the spike protein (blue) and the nucleocapsid (green) of SARS-CoV-2 were longitudinally measured since time point of hospitalization. Curves were calculated using non-linear fitting.

Figure S5: Anti-SARS-CoV-2 IgM responses of individual COVID-19 patients. IgM responses of individual COVID-19 patients ($n = 25$) against the RBD protein (red) and the nucleocapsid (green) of SARS-CoV-2 were longitudinally measured since time point of hospitalization. Curves were calculated using non-linear fitting.

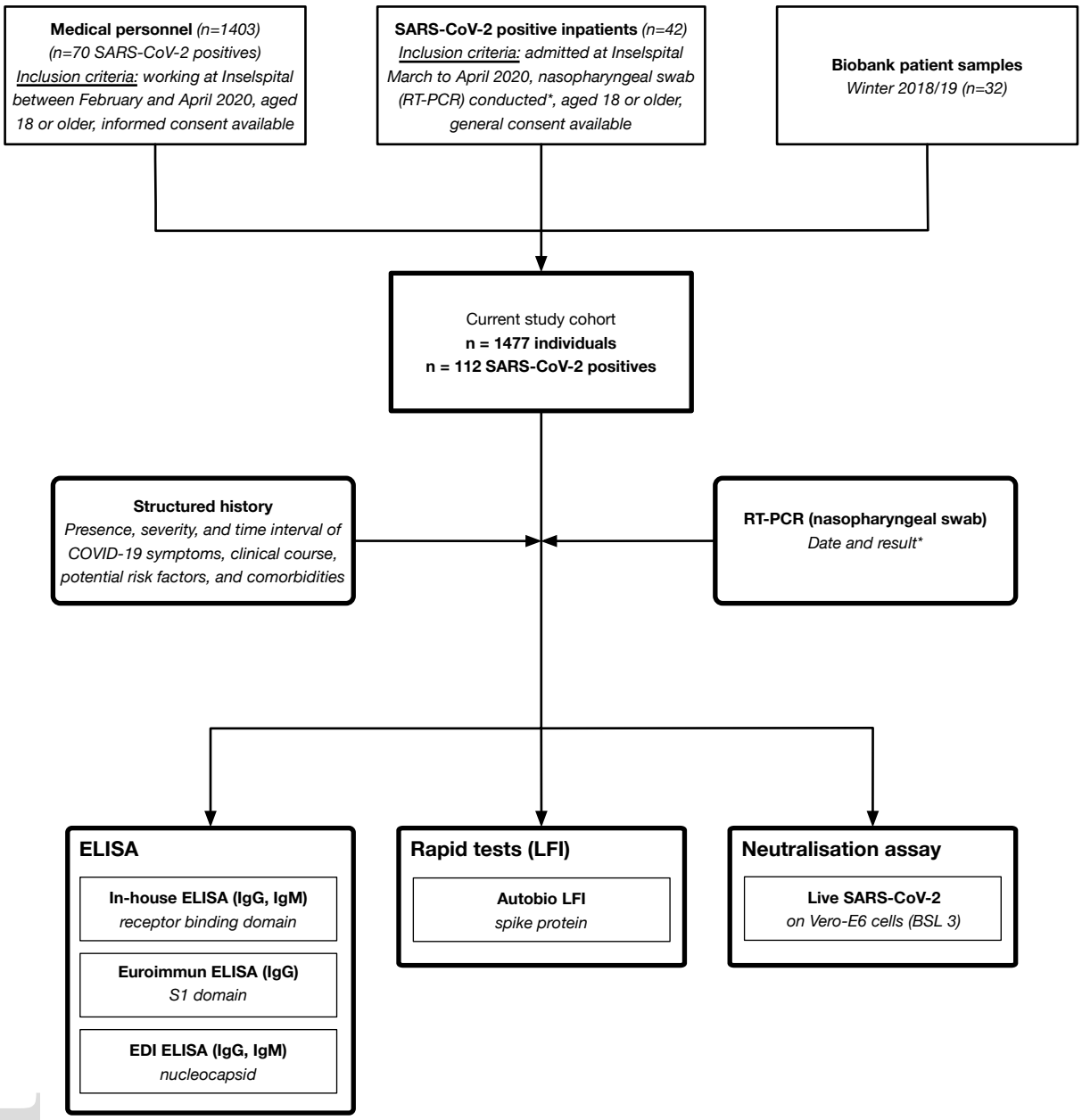
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Figure S6: Live SARS-CoV-2 neutralization. (a) Representative pictures for the neutralizing activity of a COVID-19 negative and two COVID-19 positive sera in different dilutions. (b) Virus and cell controls without virus are shown. Individual IgG and IgM antibody responses (grey dots) against RBD (c) and N protein (d) in sera of 54 COVID-19 positive and 6 COVID-19 negative individuals as measured by ELISA are shown together with the corresponding serum dilution at which full neutralization of SARS-CoV-2 is observed. Non-linear curve fitting was calculated based on the means of each serum dilution group (red circles).

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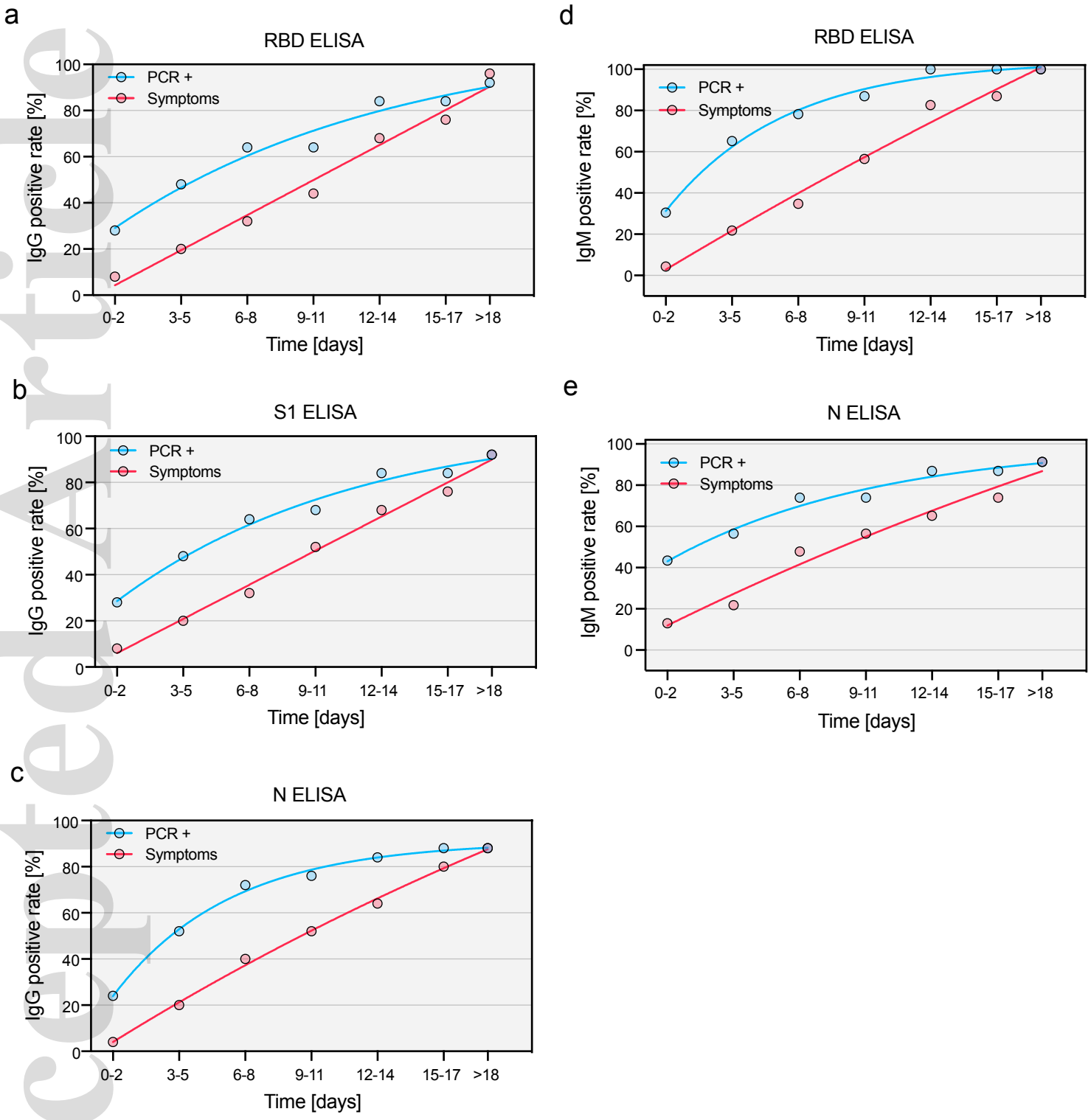
Figure 1



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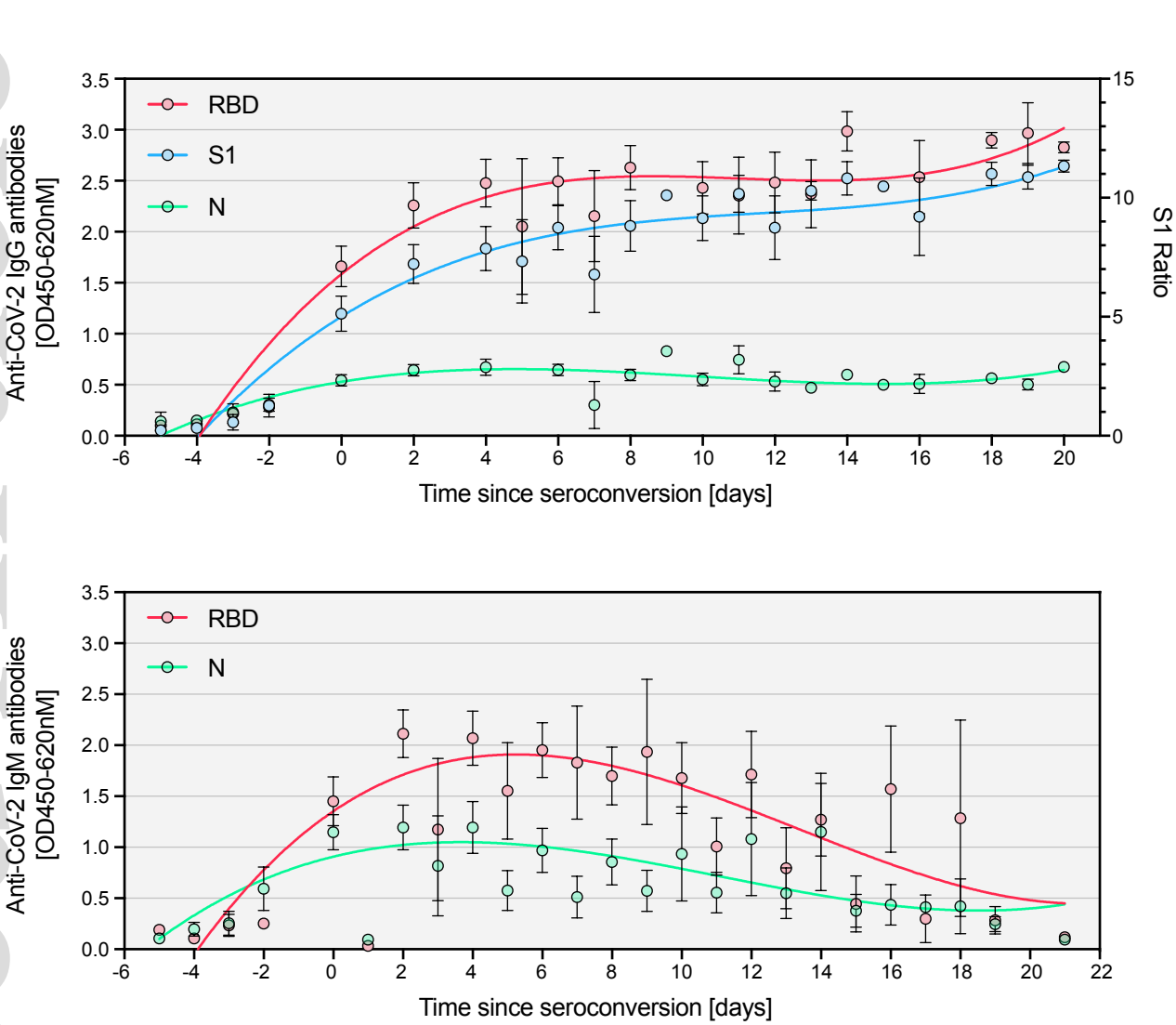
Figure 2



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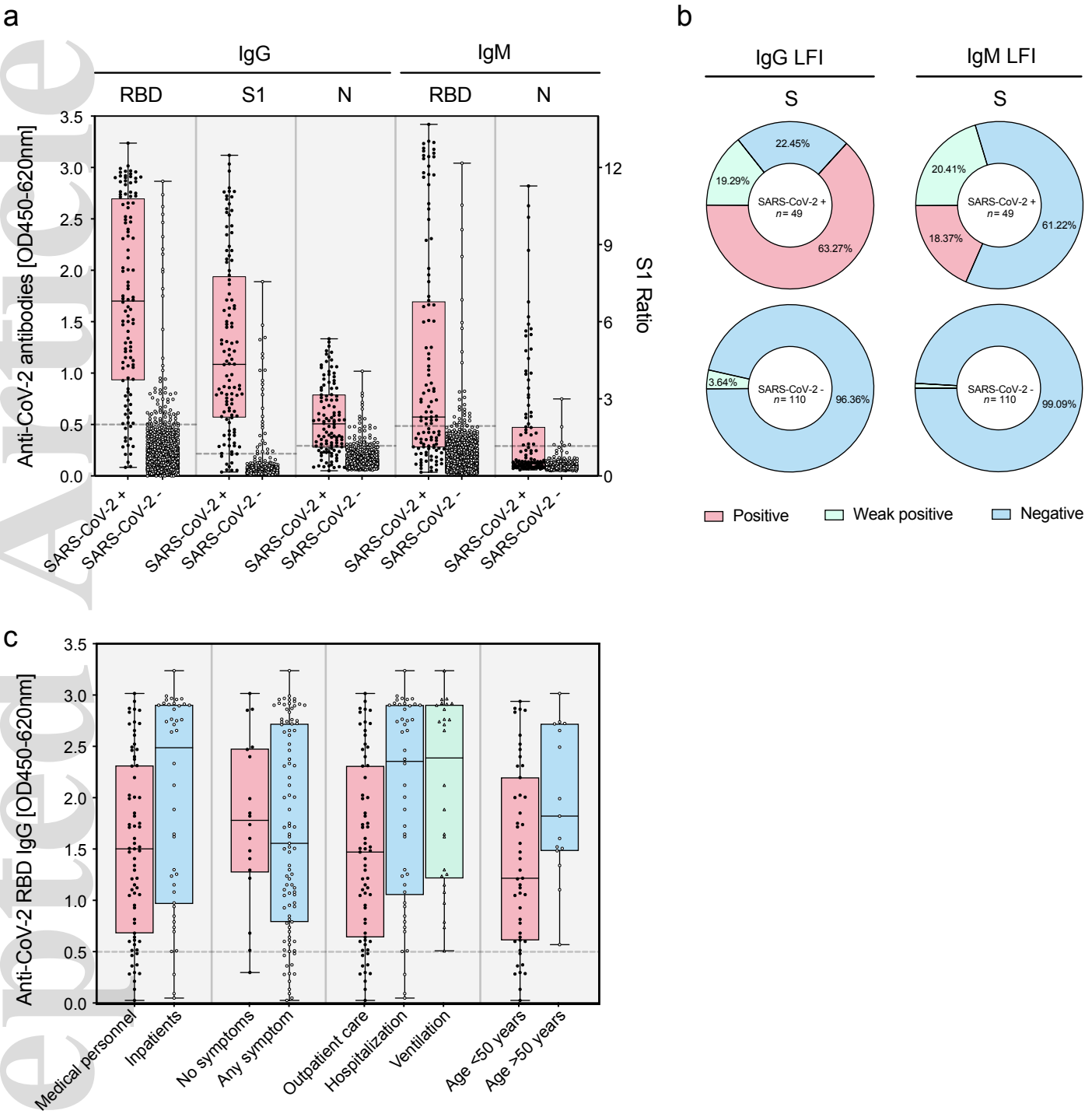
Figure 3



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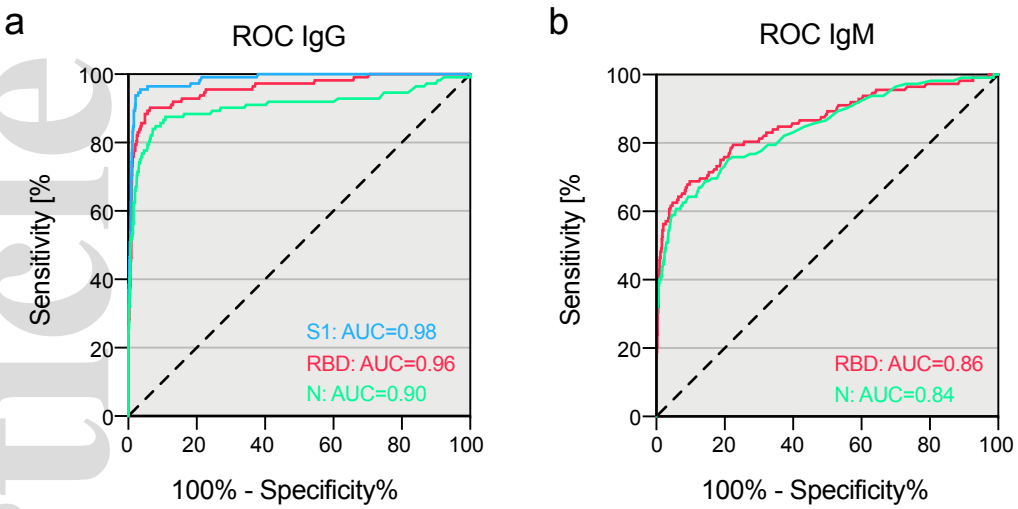
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Figure 4



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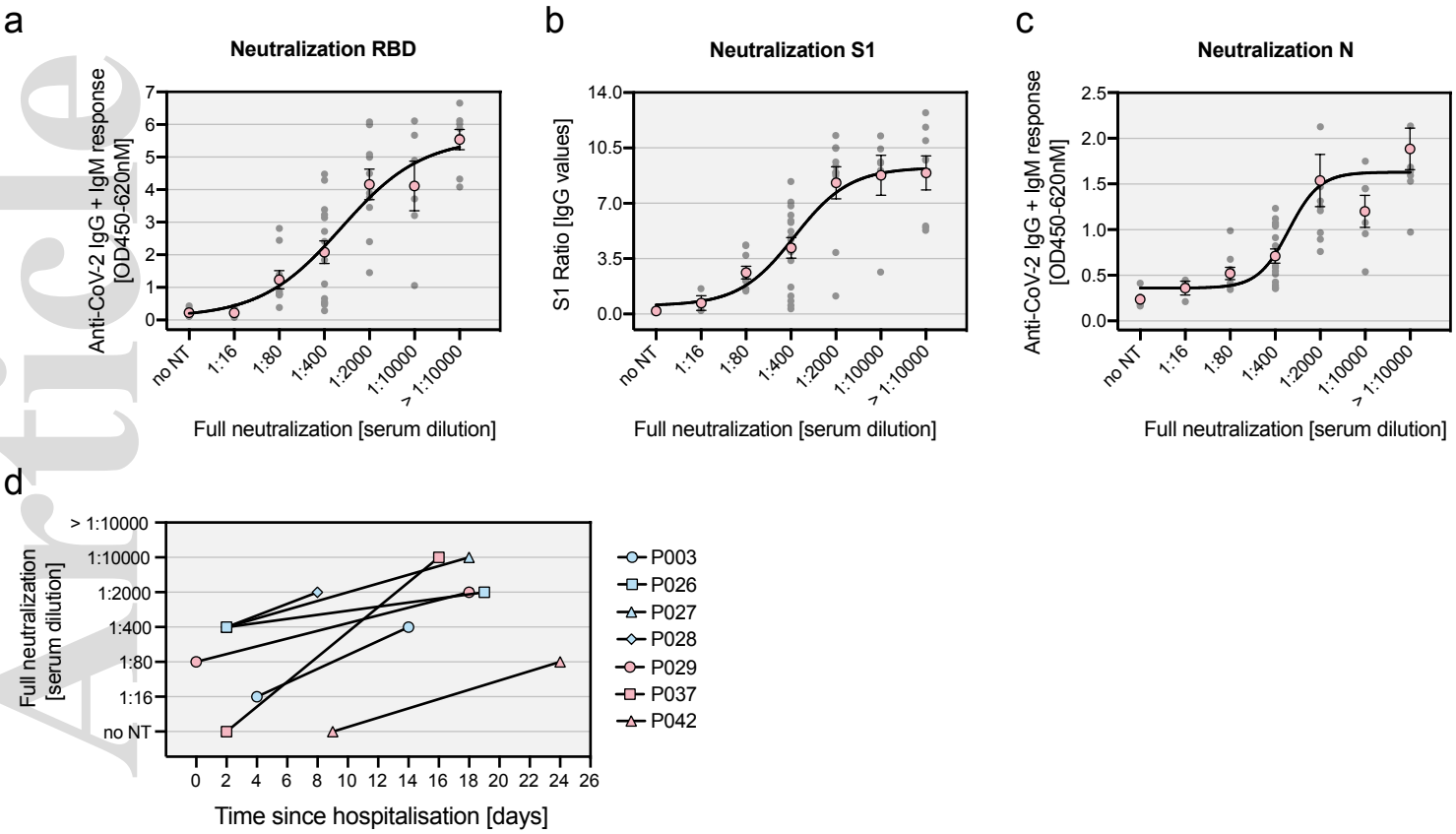
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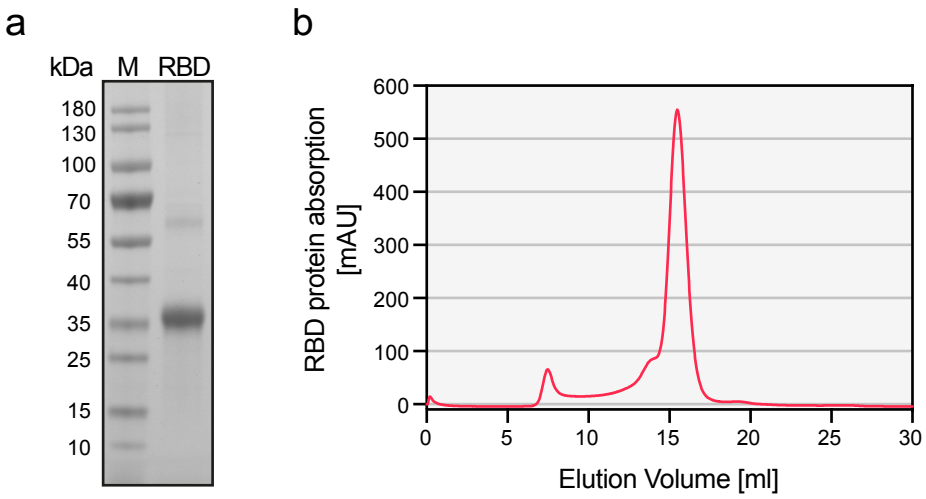
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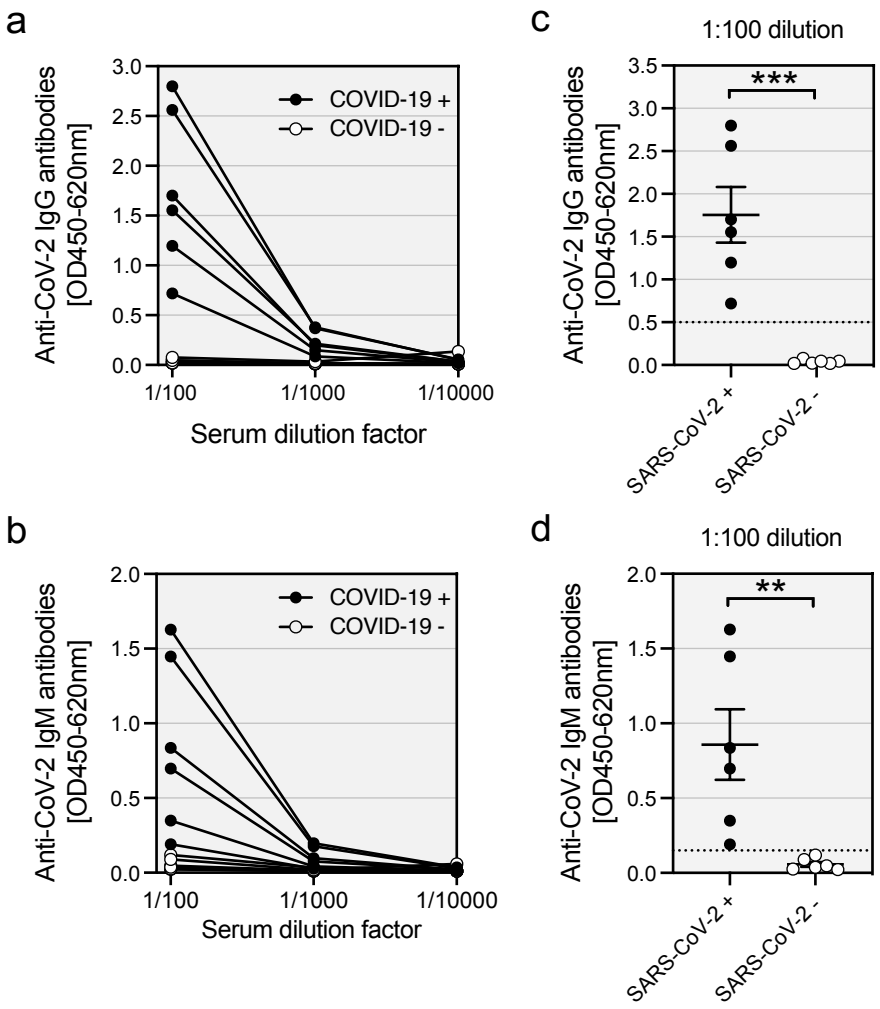
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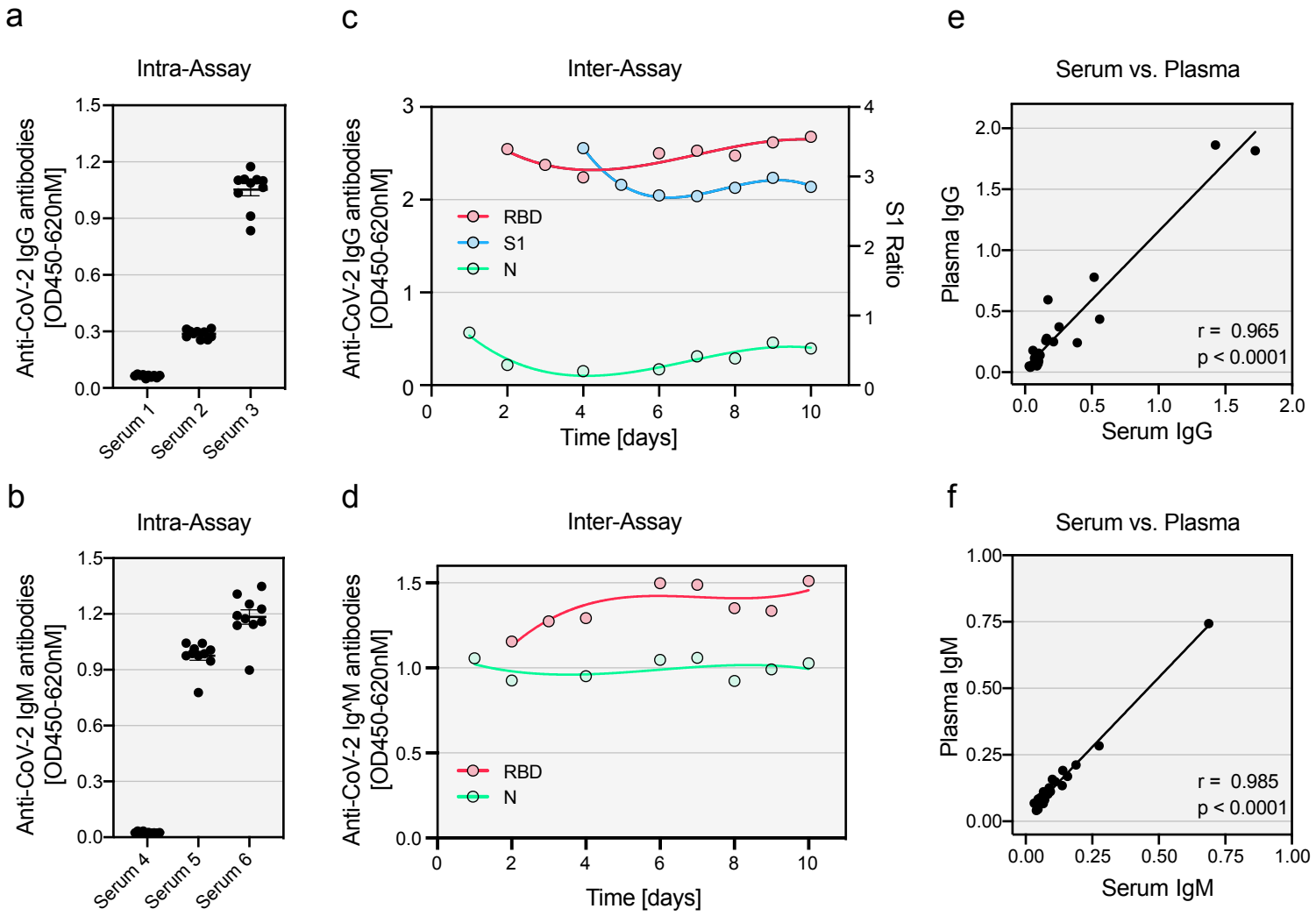
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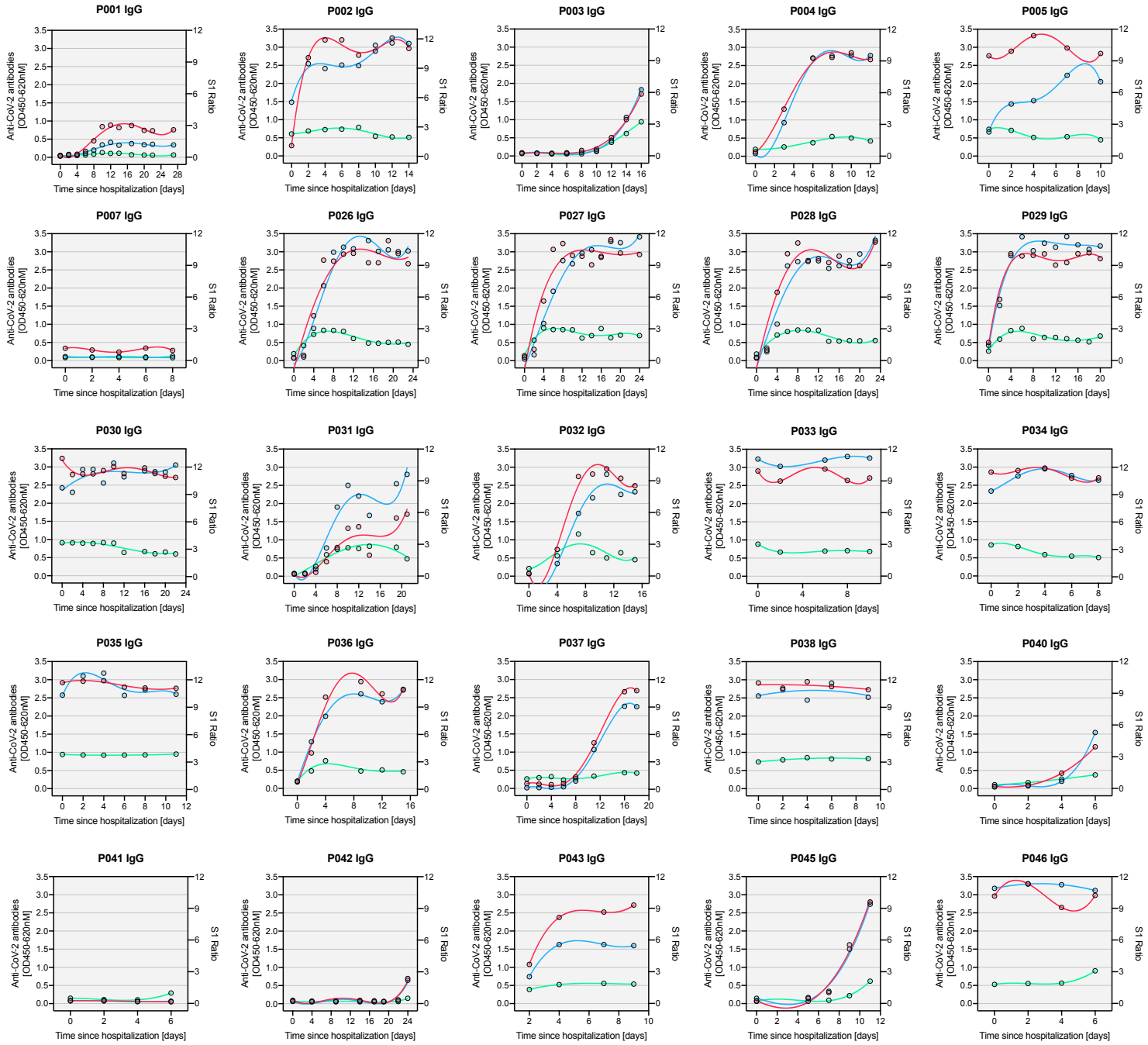
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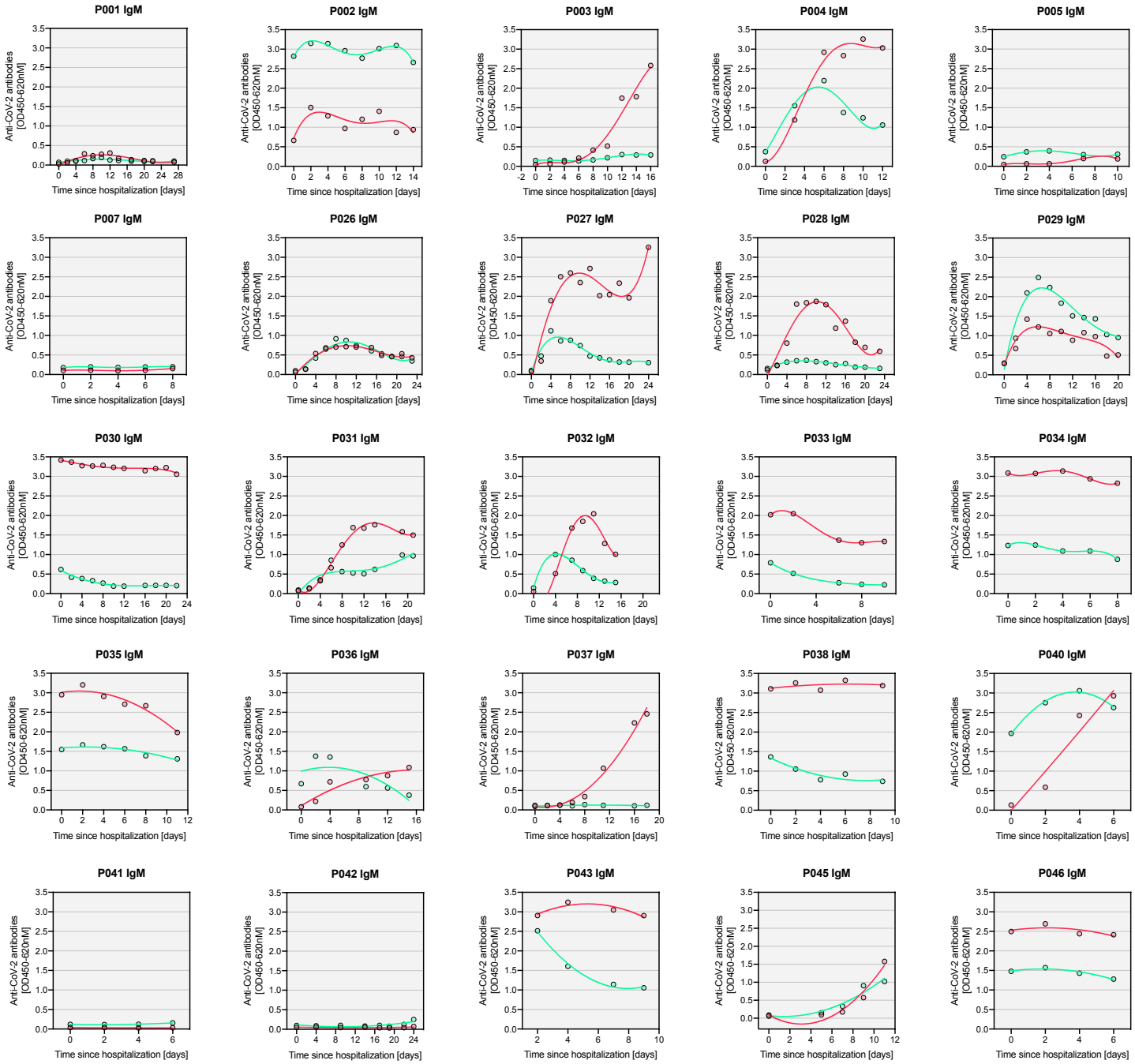
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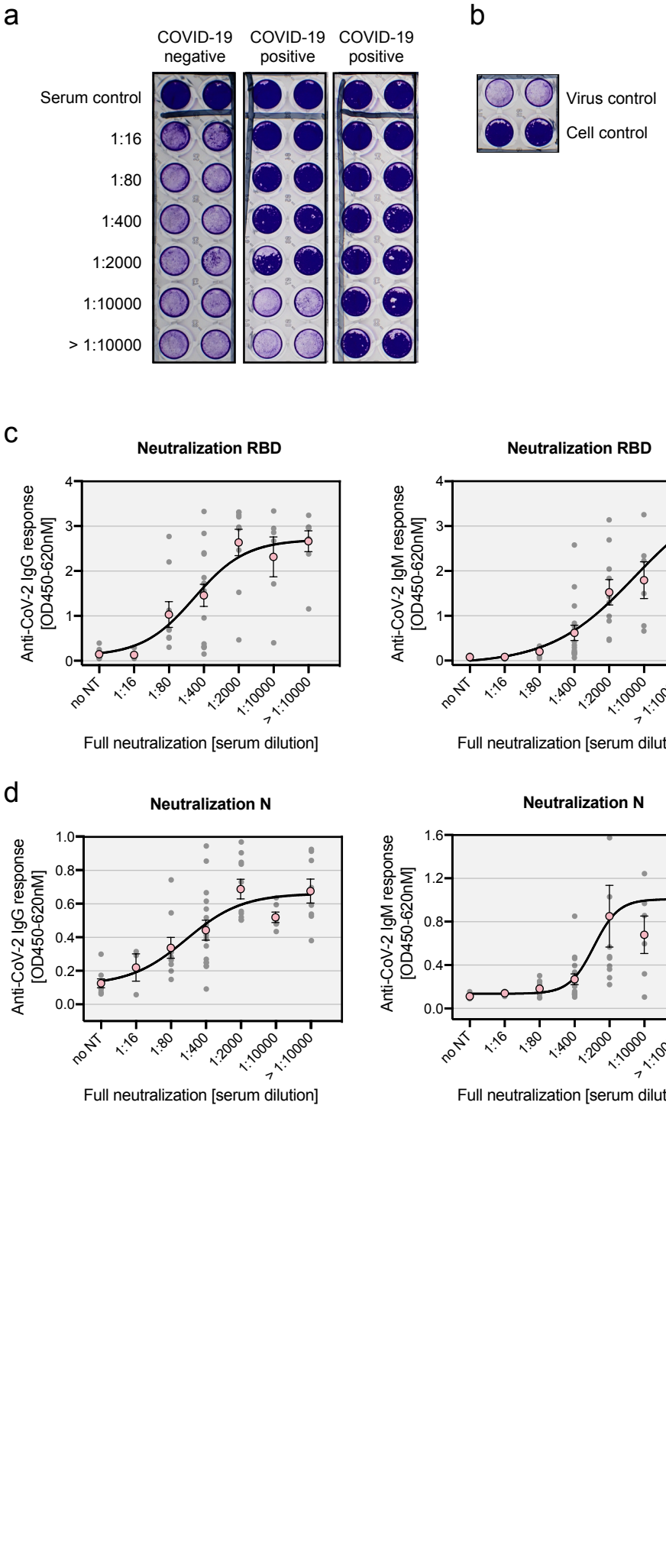
Suplementary Figure 4



Suplementary Figure 5



Suplementary Figure 6



Supplemental Tables.

Supplemental Table 1: Characteristics of inpatients.

Characteristics	*Numbers (%) or median (range)*
Numbers of patients/ samples*	42 (100) / 215 (100)
Female gender	9 (21)
Age *(years)*	66 (24, 86)
Fever	22 (52)
Pneumonia	34 (81)
ICU** admission	20 (48)
Mechanical ventilation	17 (40)
Period of hospitalization *(days)*	15 (6, 27)
Days of symptoms before hospitalization	7 (0, 18)
Days since RT-PCR	1 (0, 13)

*The first forty-two consecutive inpatients with confirmed SARS-CoV-2 infection (as defined by a RT-PCR positive nasopharyngeal swab) with more than 4 days of residual material were included. **ICU = intensive care unit

Supplementary Table 2a. Intra-Assay Precision IgG

	Sample	Mean	SD	%CV
IgG	Serum 1	0.064	0.008	11.98%
	Serum 2	0.287	0.022	7.60%
	Serum 3	1.053	0.102	9.73%

Supplementary Table 2b. Intra-Assay Precision IgM

	Sample	Mean	SD	%CV
IgM	Serum 4	0.026	0.004	15.37%
	Serum 5	0.975	0.076	7.75%
	Serum 6	1.184	0.122	10.34%

Supplementary Table 3a. Day-to-day precision IgG

	Sample	Mean	SD	%CV
IgG	OD (RBD)	2.496	0.137	5.50%
	Ratio (S1)	2.915	0.235	8.06%
	OD (N)	0.32	0.145	45.24%

Supplementary Table 3b. Day-to-day precision IgM

	Sample	Mean	SD	%CV
IgM	OD (RBD)	1.363	0.127	9.30%
	OD (N)	0.998	0.058	5.79%

Supplementary Table 4. Diagnostic accuracy of serological immunoassays for SARS-CoV-2 antibodies

Characteristics	**IgG**				**IgM**		
	ELISA			LFI	ELISA		LFI
	RBD	S1	N	N	RBD	N	N
Numbers of samples	1477	1477	1476	159	1477	1474	159
True positives	99	100	81	31	63	39	9
False negatives	13	12	30	18	49	73	40
False positives	71	23	59	4	28	9	1
True negatives	1294	1342	1306	106	1337	1362	109
AUC *(95% CI)*	0.96 (0.93, 0.98)	0.98 (0.98, 0.99)	0.90 (0.86, 0.95)	-	0.85 (0.81, 0.90)	0.84 (0.80, 0.89)	-
Sensitivity *(percent; 95%CI)*	88.4 (81.0, 93.1)	89.3 (82.2, 93.8)	72.9 (64.0, 80.0)	63.3 (49.3, 75.3)	56.3 (47.0, 65.1)	34.8 (26.6, 44.0)	18.4 (10.0, 31.4)
Specificity *(percent; 95%CI)*	94.8 (93.5, 95.9)	98.3 (97.5, 98.9)	95.7 (94.5, 96.6)	96.4 (91.0, 98.6)	98.0 (97.1, 98.6)	99.3 (98.8, 99.7)	99.1 (95.0, 100.0)
Positive likelihood ratio	17.0	53.0	16.9	17.4	27.4	52.7	20.2
Posterior probability* *(percent)*							
Positive test	58	81	58	88	69	81	90
Negative test	1	1	2	14	4	5	27